White Mutants in Mice Shedding Light on Humans

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In this article we describe the rapid advances made in the molecular genetics of three inherited pigmentation disorders: albinism, piebaldism, and vitiligo, all of which throw light on normal pigment cell function. The focus is on studies in mice, with comparison of data in humans. The critical role of tyrosinase (*c*-locus or human tyrosinase protein) in normal pigmentation and albinism has been reinforced by the cloning and identification of mutations in tyrosinase and two other melanocyte-specific oxidoreductases structurally related to but functionally different from tyrosinase: the *(b) brown*-locus protein/gp75/catalase *B* and dopachrome tautomerase. Each possesses a distinct enzyme activity and yet the three share homology in strategic regions. Most of the point mutations that reduce or abrogate the respective enzyme activities are located in those regions. Tyrosinase-negative albinism is caused only by defects in tyrosinase. A locus for human tyrosinase-positive albinism has been recently mapped to chromosome 15q11.2 → q12, at a gene identified in mice as *pink-eyed dilution*. On the other hand, several genes encoding proteins critical for the proliferation of melanocytes are known to control the piebald phenotype. So far identified are two membrane-receptor tyrosine ki ases, c-Kit and PDGF-R/α, and the ligand for *c-kit,* MGF (mast-cell growth factor, also known as stem-cell factor, c-Kit-ligand, or steel factor). Mutations in W/*c-kit (white spotting), Ph/Pdgfr/a (patch),* and *Sl/MGF (steel),* lead to a reduction in receptor kinase activity and failure of melanocytes to thrive a d reach the skin during embryogenesis. Finally, mouse mutant models suggest at least two possible causes for vitiligo, a progressive loss of pigmentation that occurs after birth. In one mutant, the B^{lt} *(light)* mouse, the cyclic death of hair melanocytes may be due to the toxicity of intermediates and byproducts of melanogenesis in the presence of a dysfunctional *b*-locus protein. In the other model, the "vitiligo mouse," in which the allele *vit* has been assigned to the microphthalmia *(mi)* locus, the loss of melanocytes may be caused by defective signal transduction, because in addition to vitiligo mi^{vit}/mi^{vit} mice have extensive piebaldism. *J Invest Dermatol 100:176S–185S, 1993*

Pigmentation in mammals is determined by melanin and depends on pigment synthesis by melanocytes as well as transfer and redistribution of melanosomes to keratinocytes of epidermis and hair. Melanosomes contain melanocyte-specific enzymes and proteins whose function is gradually becoming clarified. The complexity of control of the pigmentary system is examplified by the gradations seen in the color of skin, hair, and eyes in humans and the multitude of coat color variations in animals. In mice, more than 50 genetic loci affecting pigmentation had been described by 1979 [1] and others have been identified since. These loci can be grouped into three major, not necessarily mutually exclusive, classes: 1) those controlling the amount and/or kind of melanin produced (eumelanin and pheomelanin); 2) those affecting the migration, proliferation, and survival of melanocytes; and 3) those reflected in the shape and ultrastructure of melanocytes. This review focuses on the first two classes that are represented by albinism, piebaldism, and vitiligo. Recent reviews [2,3] provide further information on this subject. This review updates an article published in *Pigment Cell Research* [4].

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Abbreviations:
cAMP: cyclic adenosine monophosphate
cDNA: complementary deoxyribonucleic acid
CREB: cyclic-AMP responsive element binding
DHICA: 5,6-dihydroxyindole-2-carboxylic acid
DT: dopachrome tautomerase
Flt3/Flk1: fms-like tyrosine kinase 3/fetal liver kinase 1
KDR: kinase insert domain-containing receptor
L-dopa: L-dihydroxy-phenylalanine
MGF: mast-cell growth factor
mRNA: messenger ribonucleic acid
OCA: oculocutaneous albinism
TRP: tyrosinase-related protein
TYR: human tyrosinase

ALBINISM

Albinism is a generalized absence or drastic reduction of pigment in otherwise normally distributed pigment cells, including those of the eye. Striking advances have been made in the area of genes affecting melanogenesis, more specifically, the genes for tyrosinase, the *brown*-locus protein, and dopachrome tautomerase (DT). Significant homologies in the amino acid sequences suggest that these three melanosomal oxidoreductases have originated from a common ancestor gene, even though they are located on different chromosomes. However, tyrosinase has emerged as the only enzyme absolutely necessary for melanin pigmentation and tyrosinase dysfunction as the only cause of tyrosinase-negative oculocutaneous albinism (OCA). The other two enzymes modify the kind and amount of melanin produced and, in the case of the *b*-locus protein, can also affect the viability of melanocytes. Our aim in discussing these three enzymes together is to show that the tyrosinase mutations identified in OCA in both humans and mice are located in regions highly conserved among the three proteins, implying a common three-dimensional structure critical for the normal function of these enzymes.

Cloning of cDNAs and Chromosomal Mapping of Tyrosinase, the *b*-Locus Protein and Dopachrome Tautomerase

Four melanocyte-specific gene products, tyrosinase, *b*-locus protein,

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dopachrome tautomerase, and Pmel 17, were cloned by screening normal or malignant melanocyte cDNA expression libraries with antibodies raised in rabbits against hamster tyrosinase by Dr. S. Pomerantz [5–9]. The reason for the multiple recognition by the polyclonal antibodies may be impurity of the original preparation used in the immunization, because these proteins are presumed to be present as a complex in the melanosomal limiting membrane [10]. An alternative explanation is that the immunogen was tyrosinase but the unglycosylated proteins produced by the bacterial expression libraries expose shared epitopes recognized by the antibodies. In support of the latter possibility is our inability to reveal any protein other than tyrosinase in immunoprecipitation and immunoblotting experiments with extracts from normal and albino human, mouse, and chicken melanocytes [11–16].

The deduced amino acid sequences show that the three enzymes are glycoproteins of similar size, with a putative signal peptide, membrane-spanning domain, and presumptive catalytic regions, with conserved positions of histidine and cysteine, some of the sites for glycosylation, and an overall identity of 40% [5–9,17–22]. Tyrosinase was confirmed to be encoded by a single gene in both mice and humans [5,18,20–22]. Mouse tyrosinase maps to the *c*-locus on chromosome 7 [5,20,22,23], and human tyrosinase *(TYR)* to chromosome 11, region q14 → q21 [23]. The mapping of the structural gene for mouse tyrosinase to the *c*-locus on chromosome 7 has once and for all overthrown the notion that this locus encodes a tyrosinase regulatory protein [24,25]. An additional site of human tyrosinase-related sequences was detected on the short arm of chromosome 11, near the centromere (region p11.2 → cen) [23] that harbored a pseudogene. Characterization of the tyrosinase pseudogene showed that it contained only exons IV and V because only these two were recognized and amplified by the polymerase chain reaction [26]. The clone originally isolated by Shibahara *et al* [17] as tyrosinase is identical to the independently cloned TRP1 (tyrosinase related protein 1) [6], was mapped to the *brown* locus on mouse chromosome 4 [6,27] and to human chromosome 9 [28]. The murine *b*-locus protein is identical to human gp75, a highly abundant melanocytic glycoprotein [12,29]. Dopachrome tautomerase, another abundant melanocyte-specific protein, is represented by the clone TRP2 and has been mapped to mouse chromosome 14 at the *slaty* locus [7].

Despite the significant homology between tyrosinase and the *b*-locus protein, the two genes are different in structure and promoter sequences. Whereas the tyrosinase gene contains five exons and is estimated to be longer than 70 kb [20], the gene at the *brown* locus contains seven [30] or eight [31] exons and is only 15 kb [30] or 18 kb [31] in length. The promoter regions are of similar sizes; that of tyrosinase is contained within 270 bp [32,33] and that of the *brown*-locus protein within 370 bp 5′ of the translational start site [30,31]. Surprisingly, no classical cyclic adenosine monophosphate (cAMP) responsive element binding (CREB) protein consensus sequences have been found in the tyrosinase promoter despite the increase in tyrosinase and its messenger RNA (mRNA) and another melanocyte-specific mRNA, Pmel 17, in response to cyclic AMP [8,14,19]. The 11-bp motif, 5′-AGTCATGTGCT-3′, found at positions −44 to −34, and −107 to −96, in the promoters for the *b*-locus and *c*-locus genes, respectively, was shown to be a necessary positive regulatory element in melanocyte-specific expression and was termed the *M*-box [34]. This name may not be entirely appropriate, because a construct containing two copies of the "*M*-box" upstream from the thymidine kinase promoter linked to β-Gal permitted expression of the transfected reporter gene even more strongly in non-melanocytes (JEG3 cells) than in B-16 melanoma cells [34]. Moreover, although electrophoretic band shift analyses detected a nuclear factor from B-16 melanoma cells that bound specifically to this motif, similar binding activity was found in nuclear extracts from NIH 3T3 fibroblasts [34]. The existence of an apparently *M*-box–specific nuclear activating factor in non-melanocytes in the face of the so far unique occurrence of the *M*-box sequence in two otherwise quite distinctive promoters for two melanocyte-specific genes remains a puzzling enigma.

Functions of Tyrosinase, the *Brown*-Locus Protein, and Dopachrome Tautomerase Tyrosinase is essential to melanogenesis. The enzyme catalyzes three reactions *in vitro:* hydroxylation of L-tyrosine to L-dihydroxy-phenylalanine (L-dopa) [35], and oxidations of L-dopa to L-dopa quinone [35] and 5,6-dihydroxyindole to indole-5,6 quinone [36]. Tyrosinase is sufficient for melanin synthesis *in vitro* and may suffice also *in vivo,* as shown by transfection of tyrosinase complementary DNA (cDNA) into fibroblasts, and mammary carcinoma cells, as well as into amelanotic melanoma cells and albino melanocytes, turning the non-melanocytic or amelanotic pigment cells into highly pigmented ones [21,37–41]. Also, tyrosinase minigenes injected into fertilized eggs from an albino mouse strain yielded normally pigmented transgenic mice [42,43], and a retroviral vector carrying the human tyrosinase gene into Balb/c albino embryos gave rise to patches of pigmentation in adult mice [44], indicating that the albino melanoblasts incorporated these genes and became pigmented in a normal fashion. The organelles into which the melanin has been deposited have not been identified. In the case of albinos, these are assumed to be the albino (pre)melanosomes. In the cells of non-melanocytic origin the melanin may be in lysosomes, which have an acidic internal pH that would favor initiation of melanogenesis [45], or in peroxisomes. Both human and murine tyrosinases have the presumptive peroxisomal targeting sequence, SHL [46], at the carboxy terminus [47].

In melanocytes, melanin production is modulated by mutations both inside and outside the tyrosinase locus, indicating the presence of enzymes or agents that positively or negatively regulate tyrosinase activity and/or enhance or obstruct the synthesis of melanin. Such an enzyme was suggested to be the *b*-locus protein/gp75 shown to be able to degrade H_2O_2 and thus given the tentative name catalase *B* [12]. H_2O_2 is probably formed during the catalytic conversion of tyrosine to melanin precursors and melanin [48] and, unless removed, can destroy the precursors and melanin. The *brown*-locus protein/gp75 is not another tyrosinase [49,50] because it does not have tyrosinase activity *in vitro* [12,51–53] and tyrosinase activity remains absent from amelanotic human melanoma or breast carcinoma cells transfected with the cDNA encoding the wild-type *b*-locus protein [37]. Moreover, human and murine albino melanocytes carrying mutations in the *c*-locus but possessing an active wild-type *b*-locus protein/gp75/catalase *B* have no tyrosinase activity, whereas melanocytes carrying the wild-type allele for the *c*-locus and mutant *b*-allele with a null mutation exhibit normal levels of tyrosinase activity [11,13,16].

The *brown*-locus/gp75/catalase *B* appears to be a highly antigenic glycoprotein. Monoclonal antibodies against it have been raised inadvertently in four independent laboratories by the immunization of rodents with crude mixtures of melanosomal proteins in hopes of raising antibodies against tyrosinase ([51,54,55] and personal communication by Dr. K. Jimbow, Edmonton, Alberta, Canada). This antigenicity may be due to a heme- or porphyrin-bound epitope [12] known from other systems to evoke an intense immune response [56]. Heme-containing oxidoreductases have been identified as autoantigens in autoimmune thyroiditis [57] and idiopathic autoimmune hepatitis [58], and catalase *B*/gp75 is a melanoma-associated autoantigen [29].

Dopachrome tautomerase [59] was discovered more than a decade ago as a "dopachrome conversion factor" [60,61], was cloned as TRP 2, and mapped to the *slaty* locus on mouse chromosome 14 [7]. The enzyme catalyzes the tautomerization of dopachrome to 5,6-dihydroxyindole-2-carboxylic acid (DHICA) [61] rather than conversion to 5,6-dihydroxyindole. DHICA is thought to be incorporated into melanin directly or as its indole quinone derivative [62]. The murine *slaty* point mutation predicts a single substitution of Arg^{194} to Gln in a presumptive metal-binding domain [7]. Arg^{194} is conserved between murine wild-type DT and tyrosinase [7]. As the name implies, the phenotype of *slaty* is slate gray, lighter than the black wild type, and is assumed to be due to the approximately threefold or greater reduction in DT activity causing the incorporation of 5,6-dihydroxyindole in favor of DHICA [62].

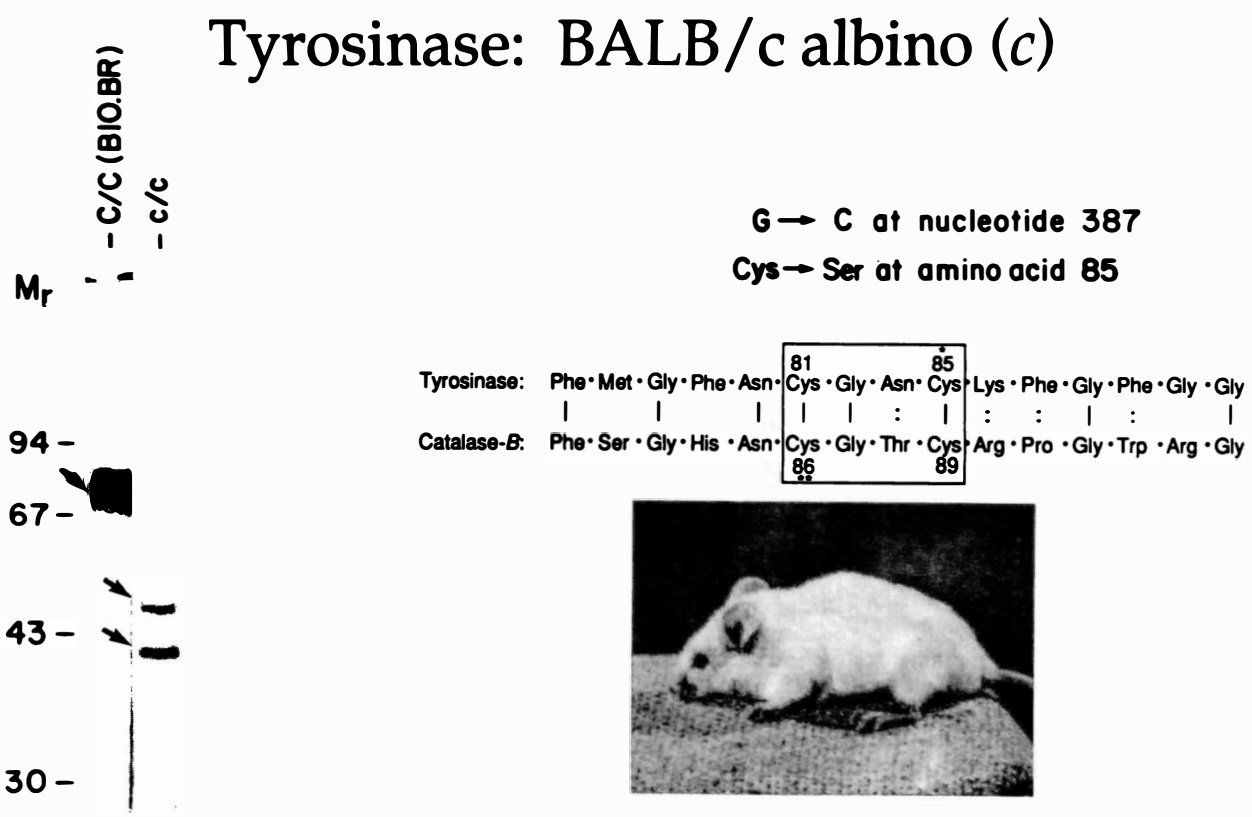


Figure 1. Murine Balb/*c* albino *(c/c)*. This *c*-locus mutation causes complete failure of pigment formation (the red eyes appear dark in this black-and-white photograph) [1]. Tyrosinase has no detectable activity because of a missense mutation ($G^{387} \rightarrow C$) substituting Cys^{85} with Ser [19,63–65] in a region highly conserved between tyrosinase, DT, and *b*-locus protein/gp75/catalase *B* (*b*-locus). Immunoprecipitated tyrosinase from cultured albino melanocytes *(left)* is low in abundance and appears mostly as degradation products [11]. Cys^{86} in catalase *B* is the site of the *b*-mutation causing substitution with Tyr [27,67].

Tyrosinase Mutations

Tyrosinase in Albino Mice: Recent molecular and biochemical analysis of melanocytes carrying mutations at the tyrosinase gene have greatly advanced our understanding of the basis for the albino phenotype. In inbred albino *(c/c)* mice, tyrosinase activity is not detectable, and the immunoprecipitated protein appears mostly as two low-molecular-weight degradation products at extremely low levels, suggesting severe sensitivity to proteolytic digestion [11] (Fig 1). Sequence analysis of the cDNA indicates a point mutation at exon 1: transition of G^{387} to C, substituting Cys^{85} with Ser [19,63–65]. These cysteine and flanking amino acids are identical with those of the *brown*-locus protein and DT, with the exception of one conservative substitution (Fig 1; in all the figures, the numbering system does not include the putative signal peptide of 18 aa, and His of tyrosinase is taken as the first residue at the amino terminus as predicted from sequence analysis and as shown by direct sequencing of the protein [66]). This segment is part of the sequence CysGlyXCys, which may be a heme- or porphyrin-binding domain [12]. In the murine *brown*-locus *b* mutation, Cys^{86} is replaced by Tyr [27,67] (Fig 1). The Balb/*c* albino mouse is homozygous for this mutation in addition to being homozygous for the albino mutation. Pigment cells from mice homozygous for the *b* mutation have little or no catalase *B* activity [12]. In homozygous *b/b* animals that have wild-type tyrosinase, the coat is brown instead of black, probably because of a lower ratio of eumelanin to pheomelanin. These examples show that point mutations in the highly conserved domains in the genes for tyrosinase, catalase *B*, and dopachrome tautomerase cause severe reduction in enzyme activity.

Another tyrosinase mutation analyzed at the nucleotide and protein levels is in the Himalayan mouse *(c^h/c^h)* (Fig 2). In himalayan melanocytes, tyrosinase activity is easily abolished by slight increases in temperature at physiologic ranges or on cell lysis *in vitro*, regardless of the method used to break down the melanocytes [11] (Fig 2). Analysis by immunoprecipitation and sodium dodecyl sulphate–polyacrylamide gel electrophoresis showed that the post-transcriptional modification of the protein, *i.e.*, the addition of carbohydrate residues, was deficient [11] (Fig 2). Sequence comparison of wild-type and mutant tyrosinase cDNAs revealed a point mutation at exon 4: transition of A^{1342} to G, which would substitute Arg for His^{402} [68]. Again, the segment of His^{402} and eight flanking amino acids is identical in tyrosinase, the *brown*-locus protein, and DT (Fig 2), suggesting a domain important for maintaining the three-dimensional structure of the proteins and/or the binding of copper (a metal critical for tyrosinase activity) or the iron in heme (a prosthetic group in many mammalian hydroperoxidases and oxidoreductases). It is thus possible that the loss of His^{402} in himalayan tyrosinase leads to structural abnormalities that reduce the efficiency of glycosylation. The reduction in carbohydrate residues, in turn, would confer instability on the protein, hence reduction or loss of activity.

Tyrosinase in Human Oculocutaneous Albinism: The sequencing of human albino tyrosinase has shown the existence of diverse but not random mutations in the tyrosinase gene. Several human albino missense mutations in tyrosinase have been identified in regions conserved between DT [7], *b*-locus protein/gp75/catalase *B*, and tyrosinase ([69–72]; reviewed in [72,73]) (Fig 3). Several frame shift mutations due to nucleotide insertions or deletions introducing early termination signals have been reported ([13,74,75]; reviewed in [72,73]). The predicted truncated protein was verified in one of these cases as an immature, smaller than normal, molecular species [13]. Likewise, the lack of recognition by antibodies directed against a 14-amino acid peptide comprising the carboxy terminal domain of human tyrosinase (anti-PEP7) [49] is in agreement with the prediction that the protein suffered truncation in this domain [13]. The melanocytes had extremely low tyrosinase activity.

Dysfunction of a gene other than tyrosinase in albinism was inferred from the observation that offspring of tyrosinase-negative

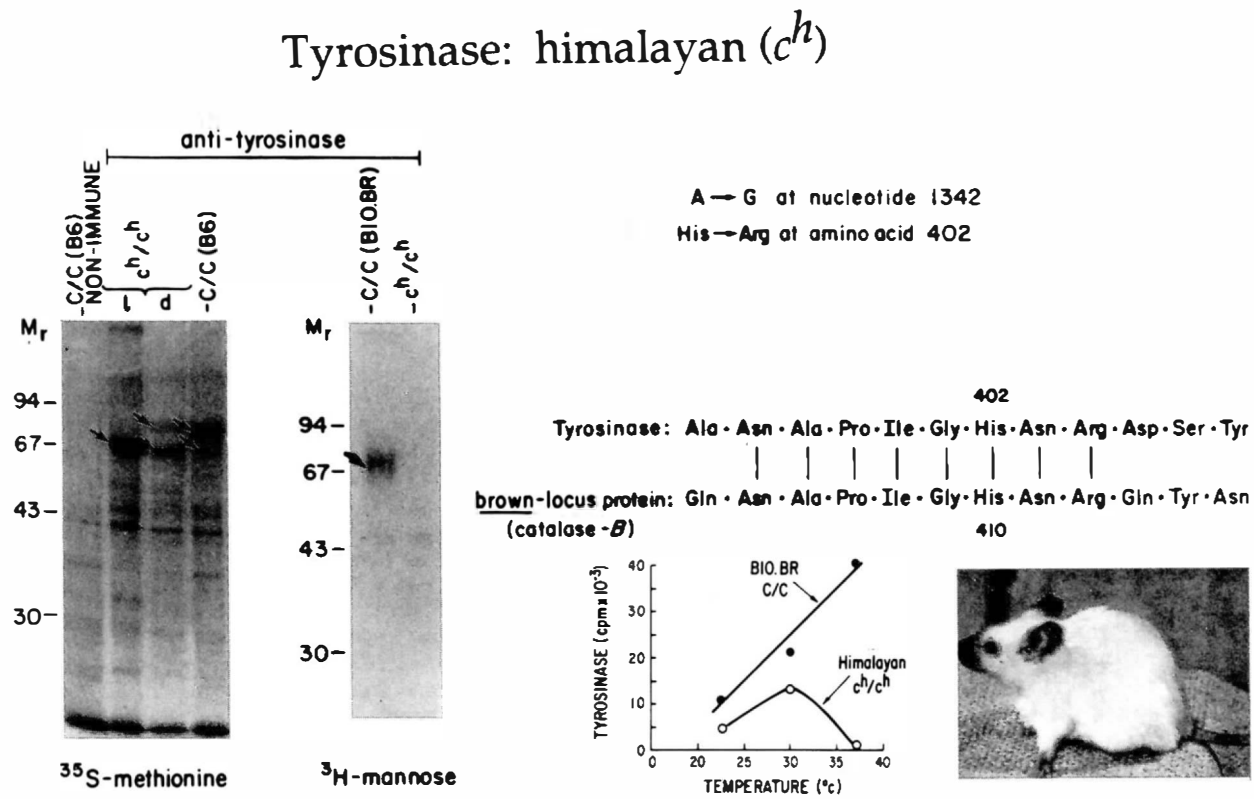


Figure 2. Murine himalayan (c^h/c^h). This *c*-locus mutation causes a white coat color except at extreme, cool body parts *(photograph)* [1]. Himalayan tyrosinase is temperature sensitive, even in the physiologic range *(graph)* [11] because of a missense mutation ($A^{1342} \rightarrow G$) substituting His^{401} with Arg [68] in a region conserved between tyrosinase, DT, and catalase *B* (*b*-locus). Gel on the left-hand side shows tyrosinase proteins *(arrows)* from cultured wild type *(C/C)* and himalayan melanocytes (l, light; d, dark himalayan melanocytes). Radioactive cell extracts were subjected to immunoprecipitation with anti-tyrosinase antibodies, electrophoresis, and fluorography. The himalayan tyrosinase appears mostly as the unprocessed species *(lower tyrosinase band)*. The protein is deficient in carbohydrate residues (right-hand gel) [11].

and tyrosinase-positive albinos were pigmented. The recently cloned murine gene *pink-eyed dilution (p)*, identified as the murine homologue of a gene mapping to human chromosomal region 15q11.2→q12 may well be such a gene [76]. This region is linked to a tyrosinase-positive oculocutaneous albinism locus [135], and a partial deletion in this gene was recently identified in an individual with tyrosinase-positive albinism [136].The predicted protein possesses 12 hydrophobic domains capable of spanning a lipid bylayer, suggesting that it is associated tightly with the melanosomal membrane. The *pink-eyed dilution* transcripts were abundant, as expected, in cultured melanocytes, and neonatal and adult eye tissue, but were present also in fetal, neonatal, and adult brain, adult testis, and ovary. Homology to bacterial proteins suggests that the *p*-encoded protein acts as a transporter for tyrosine and/or DOPA, the substrates for tyrosinase [136].

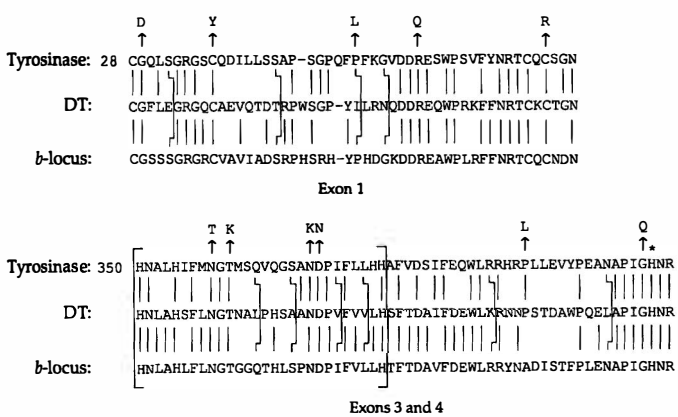


Figure 3. Tyrosinase mutations in human OCA [69–73] in regions of high homology between human and mouse tyrosinase, DT, and *b*-locus protein/gp75/catalase *B* (*b*-locus). The sequences given represent the mouse proteins as aligned by Jackson *et al* [7]. *Asterisk*, site of the mouse himalayan mutation ($His^{402} \rightarrow$ Arg). The region in *brackets* indicates a putative copper binding site.

Another mouse gene, *pallid*, whose dysfunction causes hypopigmentation due to immature and sparse melanosomes, was recently identified as the locus for erythrocyte membrane protein 4.2 [137]. The *pallid* phenotype produces defects in two other subcellular organelles, platelet dense granules and kidney lysosomes. The 4.2 pallidin protein belongs to the family of transglutaminase enzymes, but lacks transglutaminase activity [137]. This homology and the findings that it is N-myristylated and localized in melanosomes, suggests that defects in pallidin can be the cause of human diseases associated with a spectrum of storage pool deficiency such as the Hermansky-Pudlak syndrome and Chediak-Higashi disease [137].

PIEBALDISM AND VITILIGO

Piebaldism, epitomized by the white forelock in humans and white spotting in animals, is characterized by a reduction in the number of pigment cells in well-delineated patches of skin, down to complete absence. The congenital white spotting is stable and the pattern determined during embryogenesis. Vitiligo is patchy post-natal loss of pigment cells, acquired in childhood and adolescence, or even later in life, and in rare cases may progress to total depigmentation. Several mouse models exist for the study of piebaldism and vitiligo [1,77]. Mutations that result in piebaldism have been identified at minimally nine independent loci [1]. These are known as *steel* (*Sl*, chromosome 10), piebald spotting (*s*, chromosome 14), lethal spotting (*ls*, chromosome 2), belted (*bt*, chromosome 15), microphthalmia (*mi*, chromosome 6), and several that are clustered on chromosome 5, including dominant white spotting *(W)*, patch *(Ph)*, rump-white *(Rw)*, and recessive spotting *(rs)*. So far, only the *W*, *Sl*, and *Ph* mutations have been studied at the molecular level.

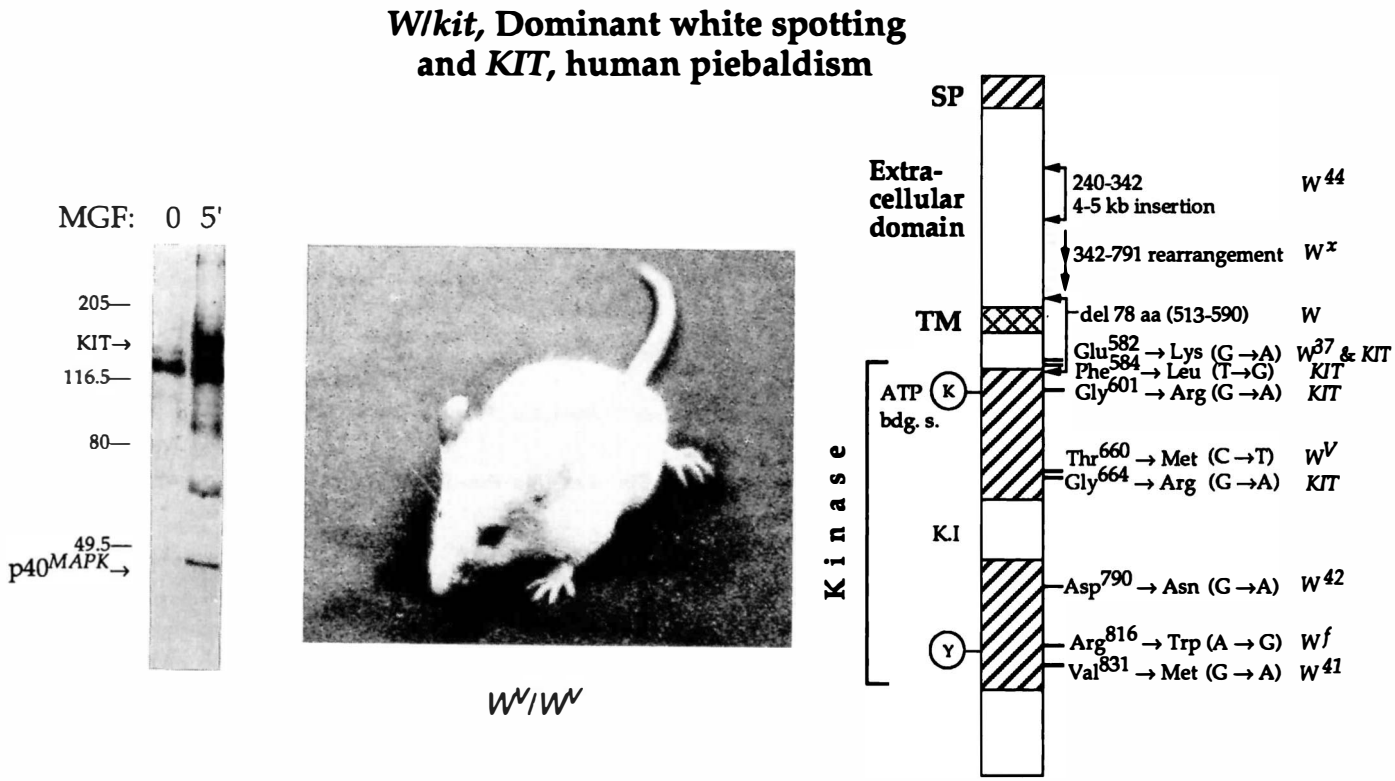


Figure 4. Murine and human piebaldism. *Left:* Western blot with anti-phosphotyrosine antibodies of cell extracts from human melanocytes deprived (0) or stimulated for 5 min (5') with MGF. The blot shows tyrosyl-phosphorylation of p145^{kit}, MAP-kinase (p40^{mapk}), and several other proteins in response to MGF [95]. *Middle:* photograph of W^v/W^v mouse [1]. *Right:* schematic representation of *c*-kit and localization of mutations in murine *(W)* and human *(kit)* piebaldism. *Hatched areas* in intracellular regions of the receptor indicate kinase domains. SP, signal peptide; TM, transmembrane domain; ATP bdg.s, ATP binding site; KI, kinase-insert. Mutations as reported [79,80,96–99,101,114–116]. Diagram adapted from [97].

Among the possible animal models for vitiligo, molecular data are available for the murine B^{lt} mutation. None exist for human vitiligo.

White Spotting, c-*kit*, and MGF Mutations at the *dominant white spotting (W)* and *steel (Sl)* loci affect the proliferation of melanoblasts as well as hemopoietic stem and primordial germ cells during embryogenesis [78]. Homozygous mice are characterized by extensive white spotting and suffer from anemia and sterility. The identification of c-*kit* and the *kit*-ligand, mast cell growth factor (MGF), as the genes at the *W* and *Sl* loci was an important step toward elucidating the mechanism by which *W* and *Sl* mutations exert their pleiotropic effects [79–88]. MGF is also referred to as the *kit*-ligand, stem-cell growth factor, or *steel* factor because it is encoded at the *steel* locus [81–88].

C-*kit* encodes a transmembrane receptor tyrosine-kinase with homology to CSF-1-R and PDGF-R (receptors for colony-stimulating factor 1 and platelet-derived growth factor) [89,90]. Ligand binding causes dimerization of the receptor and activation of the kinase through autophosphorylation, followed by a cascade of tyrosine phosphorylations on several intermediates [89,91–95] (Fig 4). Tyrosine-phosphorylated intermediates in the *c*-Kit signal-transduction pathway were identified as phosphatidylinositol (PI) 3'-kinase, Raf1, and phospholipase Cγ (PLCγ), all of which become associated with the receptor on ligand binding [91,92,94], and the serine/threonine kinase MAP kinase/ERK (microtubule-associated protein 2-kinase/extracellular signal-regulated kinases [94,95]).

The common phenotypic denominator of mutations at the *W* locus is reduced c-Kit receptor tyrosine kinase activity [79,80,96–98]. The reduction may be due to a decreased abundance of receptor protein with apparently normal kinase activity (the dominant alleles W^{44}, W^x, and W^{57}), or expression at normal levels of a defective enzyme (the dominant homozygous-lethal alleles W^{37} and W^{42}, the moderately dominant homozygous alleles W^{41} and W^{55}, and the heterozygous W/W^v). The dominant-negative characteristic of the *W* mutations was confirmed by recapitulating the W^{42} heterozygous phenotype in *c-kit*^{w42} transgenic mice [99]. In one case (W^{44}, Fig 4), reduced abundance was due to a rearrangement within the c-*kit* gene as a result of an insertion, affecting the levels of *c-kit* transcripts [79]; in another case, W^{19}, *c-kit*, and the adjacent gene *Pdgf-ra* are deleted [79,80,100]. Reduced activity is due to missense mutations causing substitutions at highly conserved positions in the kinase domain [97,98,101] (Fig 4). In the case of the W^{42}, W^{37}, and W^{41} mutations, the ligand-induced association with substrate proteins PI 3'-kinase and PLCγ is abolished or reduced, respectively [92,102]. In view of the finding that PI 3'-kinase binds to the kinase-insert domain of *c*-Kit ([103], see Fig 4), it is likely that ligand-induced *c*-Kit autophosphorylation alters the conformation of the receptor in such a way as to facilitate the association with substrates [103]. Recent studies in which anti–*c*-Kit blocking antibodies were injected into pregnant mice showed that *c*-Kit kinase was important during a brief, single-day *post-coitum* period, around day 14.5 when melanocytes proliferate in the mesoderm before entering the epidermis [104]. Injection of the antibodies at an earlier stage of development when melanocytes exit from the neural crest, or later on during embryonic life, had no effect on pigmentation. In addition, *in situ* hybridization with probes for *c-kit* and *b*-locus protein has shown that *kit* is expressed in melanocytes of day 10 embryos, at which stage the pigment cells are already in the presumptive dermis, supporting the conclusion that the receptor kinase is not critical for early migration but rather for the proliferation and survival of melanocytes in the skin [105–108]. Interestingly, the white-spotted phenotype caused by the W^v mutation is corrected in part in the inbred offspring of *ret*-transgenic W^v/W^v mice [109]. The *ret* oncogene is a constitutively active receptor-tyrosine kinase with homology to c-*kit* that apparently stimulates the same signal-transduction pathway induced by c-Kit in melanocytes.

The prediction that mutations in c-*kit* might also be the cause for human piebaldism was based on chromosome studies of piebald patients with additional gross developmental abnormalities, which

showed deletion or translocation involving chromosome 4q21 [110,111], the region to which c-*kit* has been assigned [89,90]. DNA analyses from two piebald patients (one with other abnormalities and the other otherwise normal) revealed a deletion encompassing the entire coding region of c-*kit* and involving also the closely linked gene *PDGF-R/α*, reminiscent of the murine W^{19} mutation [112,113]. Furthermore, identification of missense mutations, deletions, and duplications have demonstrated that, indeed, dysfunction of *kit* is the cause of some human piebaldism. Missense mutations include $Glu^{582} \rightarrow Lys$ (as in the murine W^{37} mutation) [114], $Gly^{664} \rightarrow$ Arg, and $Phe^{584} \rightarrow Leu$ [115,116], possibly disrupting the tyrosine kinase domain, and $Gly^{601} \rightarrow Arg$ in the putative adenosine triphosphate binding site (Bernstein *et al*, in preparation) (Fig 4). Two other piebald individuals suffered frame shifts that led to the formation of early termination signals with the predicted consequence of eliminating the kinase-inserts and kinase domains at the respective carboxyl termini [116]. These observations, and the demonstration that KIT kinase is important for the proliferation of normal human pigment cells and, possibly, maintenance of the differentiated melanocytic phenotype *in vitro* [95], support the conclusion that human melanocytes are dependent on c-KIT kinase for normal development.

Mutations at the *Sl* locus, which encodes the *Kit*-ligand, MGF, cause similar changes in phenotype in the same three cell lineages as do mutations in the *W* locus. MGF, produced by stromal cells, exists as cell-surface and soluble forms, both with equal mitogenic potency [73–75]. Melanocytes and mast cells derived from wild-type or *Sl* mutants fail to grow on *steel* fibroblasts or *steel*-derived extracellular matrix, but thrive on wild-type fibroblasts or wild-type matrix [117,118]. The absence of surface-bound MGF from *steel* fibroblasts was confirmed by the cells' inability to bind soluble c-*kit* [88]. The Sl^{d} mutation is a 4.0-kb deletion of the transmembrane and intracellular domains in the *MGF* gene [119,120]. This finding provides evidence that, *in vivo*, cell surface MGF, rather than the soluble form, has a critical role in normal development.

The *Patch/Pdgfra* Locus The *patch* locus in mice encodes the alpha subunit of the receptor for platelet-derived growth factor (PDGF-R/α), another receptor tyrosine kinase; the piebald *Ph* mutation is a deletion [100,121]. Because of the high sequence homology, structural and functional similarities, and the close topographical proximity of *patch* and c-*kit* on chromosome 5, it is possible that the two receptor kinases have evolved from tandem duplication of a single ancestor gene. The W^{19H} mutation is a deletion of both c-*kit* and *PDGF-R/α* [79,80,100,121]. The white spotting in W^{19H} mice is less pronounced than in the *W* or *Ph* mutants, suggesting that an imbalance in the expression of the respective receptors is more deleterious to melanocyte development than a reduced but balanced activity [100].

It is thus possible that receptor tyrosine kinases act in concert and that a deficiency in one reduces the efficiency of others. In support of this idea is the recurrent finding that normal human and murine melanocytes will not be sustained in culture by only one melanocyte peptide growth factor unless the medium is supplemented with cAMP, 12-O-tetradecanoyl phorbol-13-acetate, or an additional melanocyte peptide growth factor (reviewed in [122]). It appears that the second growth factor cannot be chosen at random from the list of melanocyte-stimulating peptides but that there are distinct complementary pairs, such as bFGF plus hepatocyte growth factor (HGF) [123].

Whether inactivation of the *PDGFRA* can be as deleterious to human pigmentation as it is in mice is not yet clear. Neonatal human melanocytes do not express PDGFR/α mRNA in culture [124] and do not proliferate in response to PDGF whereas murine melanocytes do (our unpublished results). Whether the PDGFRA is expressed in human melanoblasts during embryogenesis or prior to hair follicular renewal, is a subject that has not been investigated.

Together, the genetic and biologic data suggest that melanocytes depend on more than one receptor kinase during the course of embryonic development. The cloning of additional receptor tyrosine kinases may furnish clues to the nature of the gene products in other loci affecting pigmentation. For example, two new receptor tyrosine kinases, designated kinase insert domain-containing receptor *(KDR)* and fms-like tyrosine kinase 3/fetal liver kinase 1 *(Flt3/Flk1)* with a structure typical for receptors from the c-*kit* family, were cloned recently [125–127]. The *KDR* gene maps to human chromosome 4 and mouse chromosome 5 [125] and *Flt3/Flk1* to mouse chromosome 5 next to c-*kit* and *PDGF-R/α* [127]. *KDR* and/or *Flt3/Flk1* could well be the human equivalent of rump-white or recessive spotting, the two white spotting loci linked to c-*kit* on mouse chromosome 5, but Northern blot analysis of human melanocytes and three melanoma cell lines failed to detect the *KDR* and *Flt3/Flk1* messages (B. Terman and D. Birnbaum, respectively, in collaboration with R. Halaban, unpublished results), indicating that these two kinases may not play a role in human melanocytes.

Premature Graying and B^{lt} Measured against this wealth of information, knowledge of molecular events in vitiligo is minuscule. The prevalent hypothesis for the cause of vitiligo is a combination of melanocytic autotoxicity and secondary immune response. The best advanced model for this view is the Smyth chicken [128], for which no molecular data are available at this writing. The vitiligo mutation in the C57/BL Ler-*vit/vit* mouse [77] was shown to be allelic with the microphthalmia *mi* locus [129] and may, therefore, be related to a signal-transduction pathway. The *mi* mutation was suggested to be in a gene encoding a receptor-tyrosine kinase signal-transducing protein activated by both, the *c*-Kit and *c*-fms [130]. *Mi*-mutant mice are phenotypically piebald, like *W* and *Sl* mutants, and osteopetrotic like *c-fms* mutants; in addition, ectopic expression of *c-fms* can substitute for a defective *W* allele but not a defective *mi* [130].

Another murine model for early graying is the B^{lt} *(light)* mutation at the *b*-locus [110,111] (Fig 5). The phenotype produced by the *light* mutation is associated with clumping, irregular distribution, and reduction in number of melanosomes, ending in the premature death of all follicular melanocytes [131,132]. The result is a lightly pigmented hair tip on an almost white hair shaft. The mutation is a substitution of Arg^{14} by Cys near the carboxy terminus of the putative signal peptide of *b*-locus protein/gp75/catalase *B* [133]. The Arg itself is not conserved between tyrosinase and the *b*-locus protein, but the flanking Leu and two Cys residues are (Fig 5). Interestingly, this Arg is conserved between the *b*-locus and Pmel 17 proteins [9], the latter having been mapped to the *silver* locus [9]. The *silver* mutation does not reduce the amount of melanin produced but is associated with an altered morphology of the melanocytes *in vitro* (our unpublished observation). At first, we thought that the B^{lt} mutation might cause an increased sensitivity to proteolytic degradation [12]. However, in subsequent immunoblotting and immunoprecipitation studies with a panel of antibodies to *b*-locus protein/gp75/catalase *B* no structural differences between wild-type and *light* proteins were revealed. In contrast, the abnormalities in the *b*-locus *brown* mutant protein are readily detected as loss of the TMH-1 epitope (Fig 5).

Catalases in general exist as tetramers, and undenatured catalase *B* occurs as a homodimer or as part of a larger aggregate [12]. Therefore, the B^{lt} mutation may create a condition similar to that described for the c-Kit receptor tyrosine kinase: in heterozygotes the mutant protein may complex with and inactivate the normal wild-type enzyme, causing what is known as transdominant or semidominant inheritance. Addition of a cysteine in a region of catalase *B* that already possesses two of these residues may affect the folding of the enzyme onto itself and/or disturb the interaction with other melanosomal enzymes rich in conserved cysteines (suggested to be an EGF-like repeat [7]) such as tyrosinase and dopachrome tautomerase. The melano-cytotoxicity inherent in the *light* mutation may be due to toxic intermediates and byproducts of melanogenesis, because destruction of melanocytes is observed in black (C57/BL) but not albino mice carrying the B^{lt} allele [133]. In contrast to the *brown* mutant form, the B^{lt} protein retains the highly antigenic hemelike epitope [12], which once released from moribund melanocytes may invoke an autoimmune response against melanizing pigment cells, resulting in the cyclic vitiligo of B^{lt} mice.

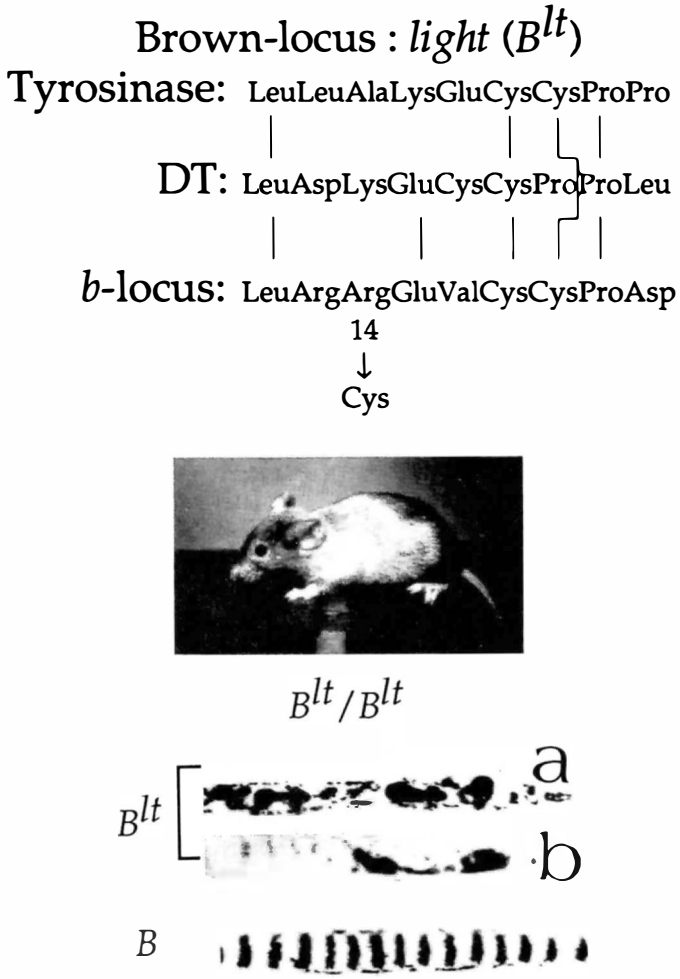
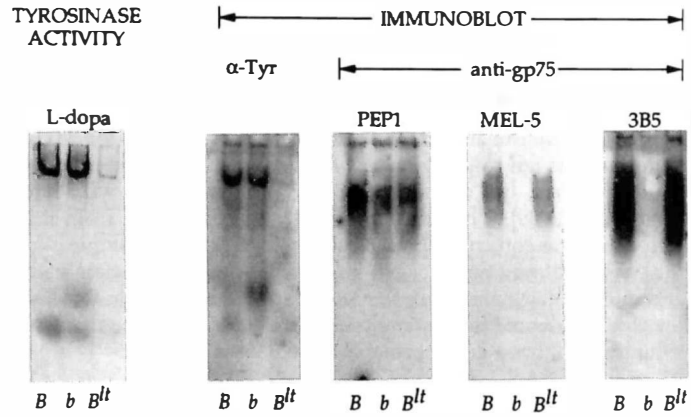


Figure 5. Murine *B-light* mutation (B^{lt}). This *b*-locus mutation causes progressive loss of melanocytes in cycling hair bulbs *(mouse photograph)* [1]. The melanocytes appear first clumped and then gradually reduced in number *(hair photomicrograph)* [131,132]. The mutation is an Arg^{14} to Cys substitution (*b*-locus sequence) [112]. The Arg itself is not conserved between *c*-, *b*-locus, and DT proteins as are several flanking amino acids. There are no gross changes in the B^{lt} catalase *B*/gp75 protein as evidenced by Western blotting with three different anti-*b*-locus protein antibodies, whereas the *b*-locus protein in *b*/*b* melanocytes lacks the TMH-1/3B5 epitope. The TMH-1 and the 3B5 monoclonal antibodies were received from Drs. V.J. Hearing and P.G. Natali, respectively. The wild-type *B*/*B* melanocytes were established in culture from B10.BR mice [11]. The *b*/*b* melanocytes were a gift from Dr. D. Bennett [134]. The cells carrying the B^{lt} mutation were grown *in vitro* from a melanoma (BULT) arising spontaneously in a B^{lt}/B^{lt} mouse [132]. Notice that the levels of tyrosinase in the BULT melanoma, determined by the dopa reaction (L-dopa) or by immunoblotting of whole cell extracts with anti-tyrosinase antibodies, are lower than in the other two non-malignant cell lines (*B* and *b*), a characteristic common to melanoma cells. If indeed destruction of melanocytes in the hair of B^{lt}/B^{lt} mice is conditioned by the presence of tyrosinase [133], then the survival of the melanoma cells *in vivo*, despite the presence of mutant B^{lt} protein, may have been enhanced by the down regulation of tyrosinase.



CONCLUSION

We have shown in this review that mutations at diverse loci, or within the same locus at diverse sites, can produce almost indistinguishable phenotypes. As a consequence, identification of human carriers of such mutations for the purpose of genetic counseling will be more complicated than in some other inherited diseases for which single genetic markers are diagnostic.

We are indebted to Dr. WK Silvers and Springer-Verlag, New York-Heidelberg-Berlin, for granting us permission to reproduce the photographs of mouse mutants, and thank Jack Schreiber for technical help.

This work was supported by NIH grants 5 R29 CA44542 and 1 RO1 AR39848 (RH).

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